Supplementary Materials

For

SIM Imaging of Bacterial Membrane Dynamics and Lipid Peroxidation During Photodynamic Inactivation with a Dual-Functional Activatable Probe

Yi Tao,[a,b] Qinglong Qiao,[*a] Yiyan Ruan,[a,b] Xiangning Fang,[a,b] Xiang Wang,[a] Yinchan Zhang,[a,b] Pengjun Bao,[a,b] Yalin Huang,[a,b] Zhaochao Xu,[*a,b]

*Corresponding author. Email: qqlqiao@dicp.ac.cn, zcxu@dicp.ac.cn

[a]CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China.
[b]University of Chinese Academy of Sciences, Beijing 100049, China.

Contents

1 Materials and Methods

1.1 Materials and instruments

All reagents were purchased from commercial suppliers (Innochem, J&K, Aladdin and Sigma-Aldrich), and used without further purification unless otherwise stated. Solvents [dimethyl sulfoxide (DMSO), dimethylformamide (DMF), methanol, ethanol, chloroform and acetonitrile] were purchased from J&K and used without further treatment or distillation. Silica gel (200-300 mesh) was purchased from Innochem.

E. coli strains were purchased from invitrogen. *Staphylococcus epidermidis* (*S. epidermidis*, ATCC 12228), *Enterococcus faecalis* (*E. faecalis*, ATCC 29212), *Staphylococcus aureus* (*S. aureus*, ATCC 25923), and *Bacillus cereus* (*B. cereus*, 1.1626) were purchased from China General Microbiological Culture Collection Center (CGMCC). *Citrobacter freundii* (*C. freundii*, ATCC 8090), and *Bacillus subtilis* (*B. subtilis*, ATCC 6633) were purchased from American type culture collection. *MDR S. aureus* strains were clinically isolated from patient of the second affiliated hospital of dalian medical university.

All ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 400 spectrometer with TMS as an internal standard. Chemical shifts were given in ppm and coupling constants (J) in Hz. High resolution mass spectrometry data were obtained with a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. UV-vis absorption spectra were collected on an Agilent Cary 60 UV-Vis Spectrophotometer. The blank correction was performed using the respective pure solvent as a reference. Fluorescence measurements were performed on an Agilent CARY Eclipse fluorescence spectrophotometer. Dynamic light scattering was performed on Zetasizer Nano (Malvern Panalytical).

Confocal images were performed on Olympus FV1000 MPE with a microscope IX 71, a 100× / NA 1.40 oil objective lens, LU-NV series laser unit (laser combination: 405 nm; 543 nm). Structured illumination microscopy (SIM) super-resolution images were taken on a Nikon N-SIM system with a 100×oil immersion objective lens, 1.49 NA (Nikon). Images were captured using Nikon NIS-Elements and reconstructed using slice reconstruction in NIS-elements.

1.2 Spectroscopic studies

The probe stock solution (2 mM) was prepared in DMSO. Samples for spectroscopic analysis were prepared through diluting the stock solution in different

solvents. The concentration of samples was usually prepared at 2 μM, unless stated otherwise.

Rhodamine B was used to obtain relative fluorescence quantum yields of compounds (fluorescence quantum yields of Rhodamine B is 0.60 in ethanol).[1] The quantum yield (φ) was calculated using the following equation.

$$\varphi F_{(X)} = \varphi F_{(S)} \cdot (A_S F_X / A_X F_S)(\eta_X / \eta_S)^2$$

Where φF, A and F represent the fluorescence quantum yield, the absorbance at the excitation wavelength and the area under the corrected emission curve, respectively. And η is the refractive index of the solvent. Subscripts X and S refer to the unknown and to the standard.

1.3 Detection of ROS in solution

DCFH as a ROS indicator was used to detect the ROS generation in solution under 561 nm laser irradiation (40.6 mW/cm^2). The fluorescence spectra of DCFH activated by **RDP** generated ROS under 561 nm laser were measured for different time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 minutes). The fluorescence spectra were measured with excitation at 470 nm and emission from 480 to 700 nm.

Using DPBF as a ROS probe, the generation of total ROS in solution under 561 nm laser irradiation (40.6 mW/cm^2) was monitored. The changes in the UV-Vis absorption spectra of DPBF were measured at different time intervals (0, 1, 3, 5, 7, 10, 13, 16, 20, 25, 30, 35, 40, and 45 minutes) following the reaction between total ROS generated under 561 nm laser irradiation and DPBF.

Using ABDA as a specific singlet oxygen ($^1O_2$) probe, the generation of $^1O_2$ in solution under 561 nm laser irradiation (40.6 mW/cm^2) was monitored. The changes in the UV-Vis absorption spectra of ABDA were measured at different time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 minutes) following the reaction between $^1O_2$ generated under 561 nm laser irradiation and ABDA.

1.4 Detection of $\cdot O_2^-$ production with EPR

Electron paramagnetic resonance (EPR) was employed to detect the generation of $\cdot O_2^-$. DMPO (5,5-dimethyl-1-pyrroline N-oxide) was used as a spin-trap agent for $\cdot O_2^-$. EPR was employed to detect the signals of the following four groups of samples: i) 100 μM of **RDP** was dispersed in methanol containing 100 mM of DMPO without light-irradiation; ii) 100 μM of **RDP** was dispersed in methanol containing 100 mM DMPO and then illuminated with 570 nm laser; iii) 100 mM of DMPO was dispersed in methanol without light-irradiation; iv) 100 mM of DMPO was dissolved in methanol

and then illuminated with 570 nm laser. The EPR signals were recorded by the Bruker A200 instrument.

1.5 Bacteria culturing, staining, and imaging

The 8 kinds of bacteria were cultured in LB liquid medium (5 mL) at 37 °C until OD_{600} is about 1.5. The bacterial cells were collected by centrifuging (10000 rpm, 2 min) and washed with PBS buffer (10 mM, pH 7.4) twice. Then the bacterial pellets were suspended in PBS solution to an absorbance of 1.0 at 600 nm.

We mixed the bacteria (1 mL) with probe solution (1 μL) and put the mixture at room temperature for 30 mins. And then we place the mixed solution into the microfluidic chip for fluorescent imaging experiment.

As for confocal imaging, the bacteria stained in the microfluidic chip were then directly imaged using confocal microscopy. Ex: 543 nm; collected: 555-655 nm for **RDP**. In co-localization experiments, DNA was labelled by DAPI. Ex: 405 nm; collected: 425-475 nm for DAPI.

As for SIM imaging, the bacteria stained in the microfluidic chip were directly imaged using super resolution microscopy.

1.6 Antimicrobial assay

Bacteria were introduced into fresh LB medium and cultured with shaking at 37 °C for 12 hours. Afterwards, 1 mL of the bacterial solution was centrifuged at 10000 rpm for 2 mins, and the bacteria were washed twice with PBS buffer (10 mM, pH 7.4) and then resuspended in PBS buffer (10 mM, pH 7.4). Then dilute the bacterial solution in PBS to a bacterial count of 10^5 CFU/mL. Mix different doses of **RDP** with the bacterial solution to obtain a mixture with staining concentrations of 0, 0.625, 1.25, 2.5 and 5 μM respectively. Divide the mixture of different concentrations into two equal parts, one part is treated under 580 nm laser (5 mW/cm^2) for 30 minutes, and the other part is left in the dark for 30 minutes. Then, apply appropriate amounts of the separately treated bacterial liquids onto LB agar plates. Place the agar plate in a 37 °C incubator for 24 hours and then count the number of colonies to obtain the killing of bacteria by the dye under different conditions.

1.7 Bacterial lipid extraction

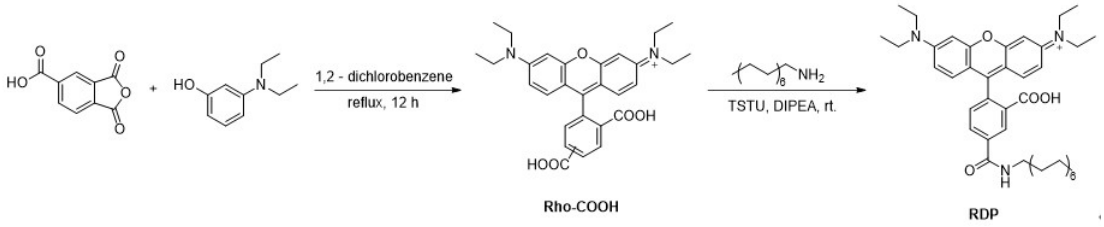
The total lipids from bacteria were extracted following the modified Bligh and Dyer method.[2] Centrifuge the treated bacterial solution to collect the precipitate, and resuspend it in 400 μL sterilized deionized water. A volume of 1.6 mL of chloroform/methanol (2:1, v/v) was added to bacterial cells. And then vortex the

mixture to mix evenly and incubated on ice for 200 min. The samples were centrifuged at 2000 rpm for 10 min at room temperature to resolve a two-phase system: an aqueous upper phase and an organic lower phase from which lipids were obtained. Collect organic phase，and the extraction was repeated twice. The extracts were dried under nitrogen flow, dissolved in 200 μL of chloroform, and stored at -80 °C.

1.8 Preparation of Bacteria Samples for SEM

Bacteria were inoculated into fresh LB medium and cultured at 37°C with shaking for 12 hours. Subsequently, 1 mL of the bacterial suspension was centrifuged at 10,000 rpm for 2 minutes, and the bacterial pellet was washed twice with PBS buffer (10 mM, pH 7.4) and then resuspended in PBS buffer (10 mM, pH 7.4). The bacterial suspension was diluted with PBS to achieve a final concentration of 10^6 CFU/mL. Three groups of 1 mL bacterial suspension were prepared: the first group was incubated in the dark without **RDP** for 30 minutes, the second group was treated with 2.5 μL **RDP** and incubated in the dark for 30 minutes, and the third group was treated with 2.5 μL **RDP** and exposed to 580 nm irradiation (5 mW/cm^2) for 30 minutes. After incubation, the bacterial suspensions were centrifuged, and the pellets were washed three times with PBS buffer (10 mM, pH 7.4). The bacterial samples were then fixed overnight with 2.5% glutaraldehyde and dehydrated by 30%, 50%, 70%, 80%, 90%, and 100% (v/v, in water) ethanol for analysis by SEM (Quanta 200F, FEI).

2 Synthesis



Scheme S1. The synthesis procedures of **RDP**.

2.1 Synthesis of Rho-COOH

Dissolve trimellitic anhydride (2 g, 10.4 mmol) and 3-diethylaminophenol (3.44 g, 20.8 mmol) in 1,2-dichlorobenzene (10 mL) under vacuum and reflux at 181°C for 12 hours. Then, the solvent was removed and the crude product was purified by column chromatography (DCM:MeOH = 10:1) to give isomers **Rho-COOH** as purple solid 1.5 g, yield 27.6%.

2.2 Synthesis of RDP

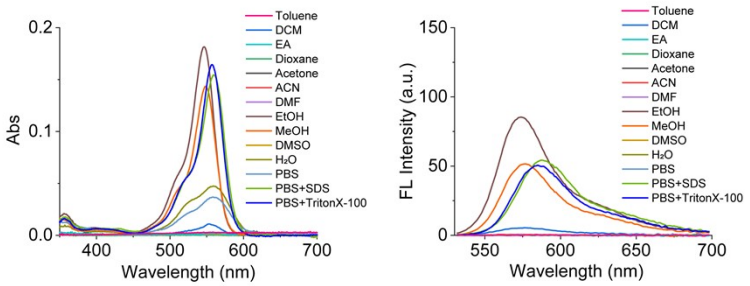
Under vacuum conditions, **Rho-COOH** (150 mg, 0.29 mmol) and TSTU (104 mg, 0.34 mmol) were dissolved in DMF (3 mL), and DIPEA (300 μL) was added to the reaction solution. After reacting at room temperature for 1 hour, the DMF solution of tetradecane (307 mg, 1.44 mmoL) was added to the reaction solution and reacted for 6 hours. Then, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (DCM:MeOH = 10:1) to give **RDP** as purple solid 15 mg, yield 7.3%.

^{1}H NMR (400 MHz, MeOD) δ 8.50 (d, J = 1.5 Hz, 1H), 8.03 (dd, J = 7.9, 1.7 Hz, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.24 (d, J = 9.5 Hz, 2H), 6.99 (dd, J = 9.5, 2.4 Hz, 2H), 6.92 (d, J = 2.4 Hz, 2H), 3.65 (q, J = 7.0 Hz, 8H), 3.43 (t, J = 7.1 Hz, 2H), 1.71 – 1.63 (m, 2H), 1.29 (t, J = 7.0 Hz, 34H), 0.89 (t, J = 6.9 Hz, 3H).

HRMS (m/z): $[M]^+$ calcd. for $C_{43}H_{60}N_3O_4^+$, 682.4578; found, 682.4625.

3 Spectral characteristics

(a)



(b)

Solvent	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}cm^{-1}$)	Δλ (nm)	Φ
Toluene	558	578	695	20	0.13
DCM	552	576	5455	24	0.75
EA	551	590	1055	39	0.20
Dioxane	553	577	275	24	0.49
Acetone	551	586	555	35	0.23
ACN	540	572	1125	32	0.27
DMF	554	582	650	28	0.21
EtOH	547	574	90710	27	0.56
MeOH	549	577	71700	28	0.45
DMSO	550	580	800	30	0.48
H_2O	559	593	23710	34	0.01

Fig.S1 (a) Absorption and emission spectra of **RDP** (2 μM) in different solvents. (b) Spectroscopic data of **RDP** in different solvents: peak UV-vis absorption wavelength (λ_{abs}), maximum emission wavelength (λ_{em}), stokes shifts (△λ), molar absorption coefficient (ε) and fluorescence quantum yield (φ).

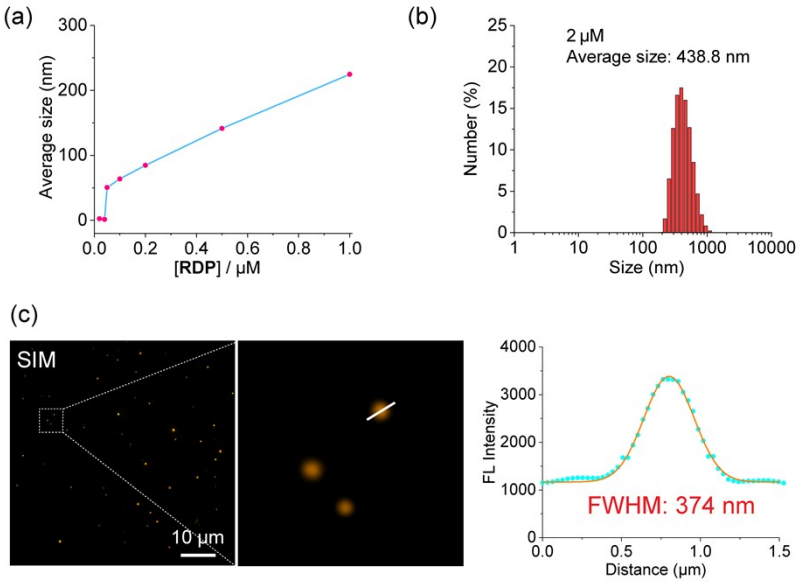


Fig.S2 (a) The average particle size of **RDP** measured by DLS varied with different concentrations (0-1 μM). (b) DLS analysis of **RDP** aggregates (2 μM) in PBS (PDI=0.337). (c) Fluorescence imaging and size analysis of **RDP** aggregates (10 μM) in ultrapure water.

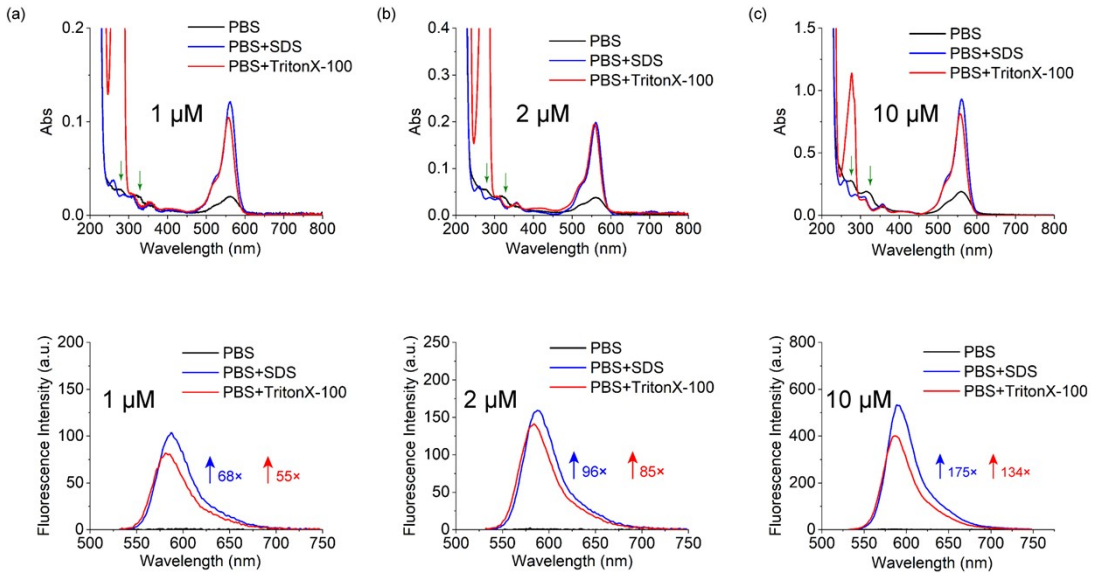


Fig.S3 UV-Vis absorption spectra and fluorescence emission spectra of **RDP** [(a)1 μM, (b)2 μM, (c)10 μM] in PBS, PBS+SDS, and PBS+TritonX-100. $\lambda_{ex/em}$ = 520/590 nm.

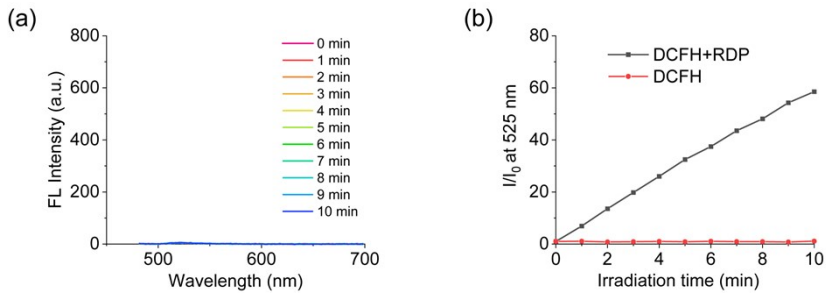


Fig.S4 (a) Fluorescence spectra of DCFH (2 μM) in PBS+TritonX-100 solution after irradiation with 561 nm laser for different time intervals. Excitation wavelength: 470 nm. (b) Plot of relative FL intensity (I/I_0) at 525 nm versus the irradiation time.

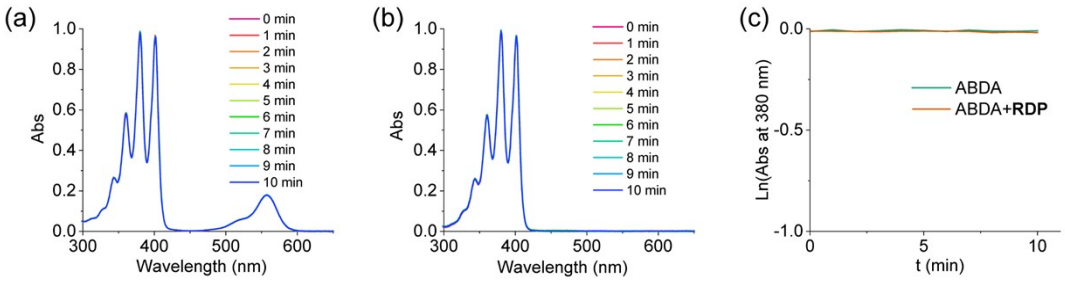


Fig.S5 (a) The absorption spectra of ABDA (80 μM) in the presence of 2 μM **RDP** in PBS+TritonX-100 solution after irradiation with 561 nm laser for different time intervals. (b) The absorption spectra of ABDA (80 μM) in PBS+TritonX-100 solution after irradiation with 561 nm laser for different time intervals. (c) Plot of the relationship between Ln(Abs) at 380 nm for ABDA and the irradiation time.

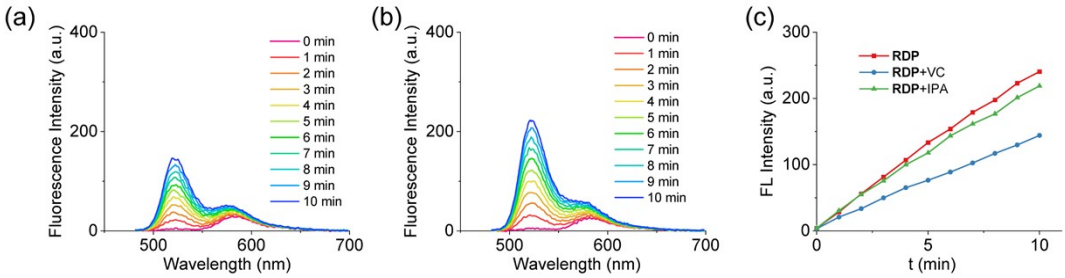


Fig.S6 (a) Fluorescence spectra of DCFH (2 μM) in PBS+TritonX-100 solution containing the $\cdot O_2^-$ quencher VC (10 mM) and **RDP** (2μM) after irradiation with 561 nm laser for different time intervals. (b) Fluorescence spectra of DCFH (2 μM) in PBS+TritonX-100 solution containing the ·OH quencher IPA (50 mM) and **RDP** (2μM) after irradiation with 561 nm laser for different time intervals. (c) Plot of FL intensity at 525 nm versus irradiation time.

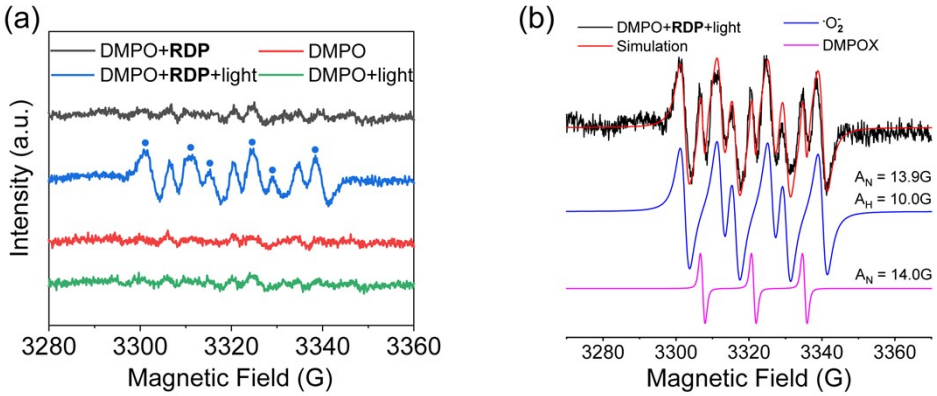


Fig.S7 EPR spectra for the detection of $\cdot O_2^-$ in methanol using DMPO as a spin trapper. (a) The reaction mixtures consisted of the following: DMPO (100 mM) with **RDP** (100 μM), DMPO (100 mM) with **RDP** (100 μM) under 570 nm laser irradiation (4 mW, 10 minutes), DMPO (100 mM) alone, and DMPO (100 mM) under 570 nm laser irradiation (4 mW, 10 minutes). (b) The fitting curve of the DMPO (100 mM) with **RDP** (100 μM) experimental group under 570 nm laser irradiation (4 mW, 10 minutes).

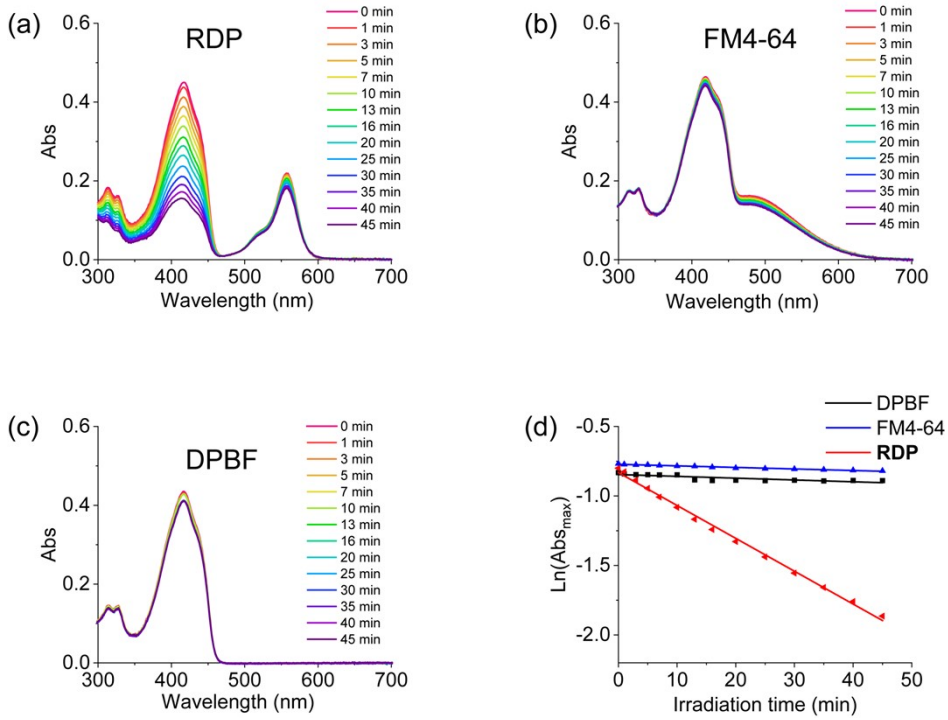


Fig.S8 UV-Vis absorption spectra of (a) DPBF (20 μM) and **RDP** (2 μM), (b) DPBF (20 μM) and FM4-64 (2 μM), (c) DPBF (20 μM) in PBS+TritonX-100 solution after irradiation with 561 nm laser for different time intervals. (d) The relationship between $Ln(Abs_{max})$ and irradiation time.

4 Imaging data supplement

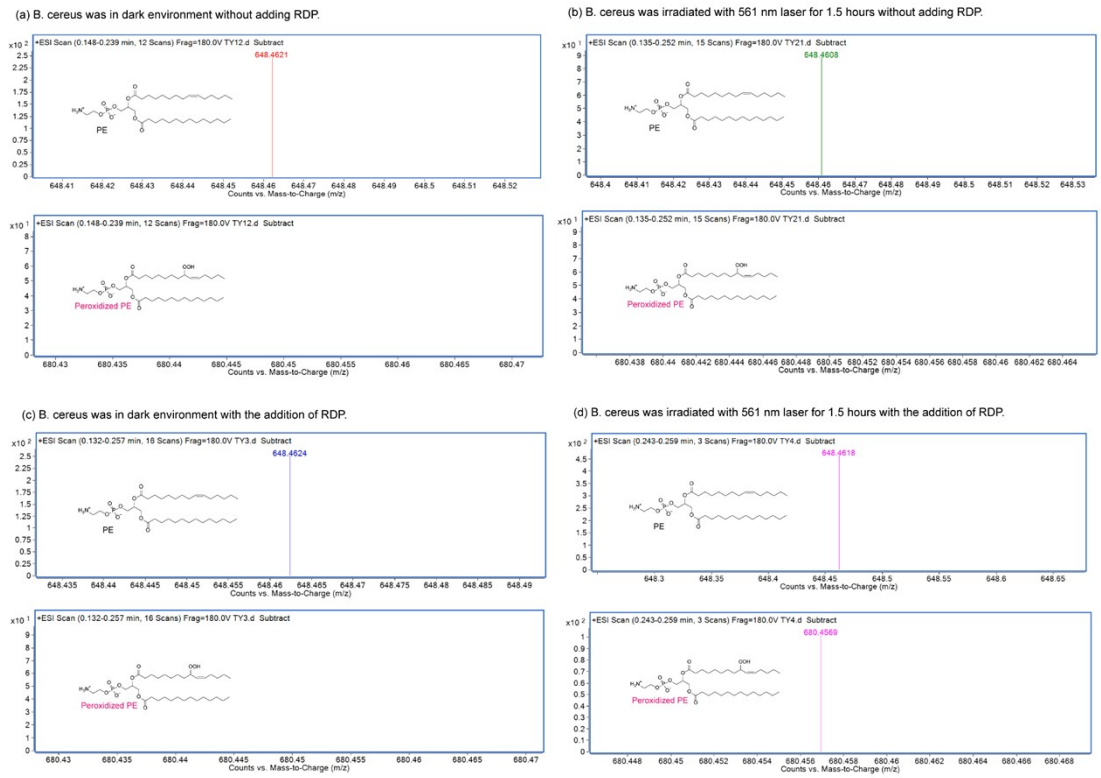


Fig.S9 HRMS analysis of raw data of PE and peroxidized PE in the plasma membrane of *B. cereus* before and after **RDP** photodynamic inactivation. (a) *B. cereus* was in dark environment without adding **RDP**. (b) *B. cereus* was irradiated with 561 nm laser for 1.5 hours without adding **RDP**. (c) *B. cereus* was in dark environment with the addition of **RDP**. (d) *B. cereus* was irradiated with 561 nm laser for 1.5 hours with the addition of **RDP**.

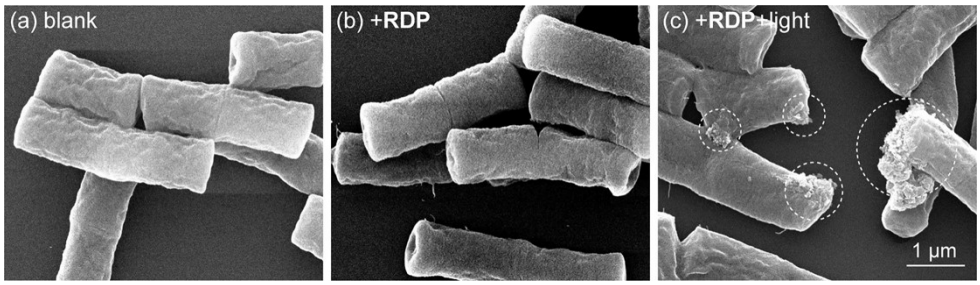


Fig.S10 SEM images of the plasma membrane of *B. cereus*. (a) *B. cereus* was in dark environment without adding **RDP**. (b) *B. cereus* was in dark environment with the addition of **RDP** (5 μM). (c) *B. cereus* was irradiated with 580 nm laser for 30 mins with the addition of **RDP** (5 μM).

5 Spectra characterization

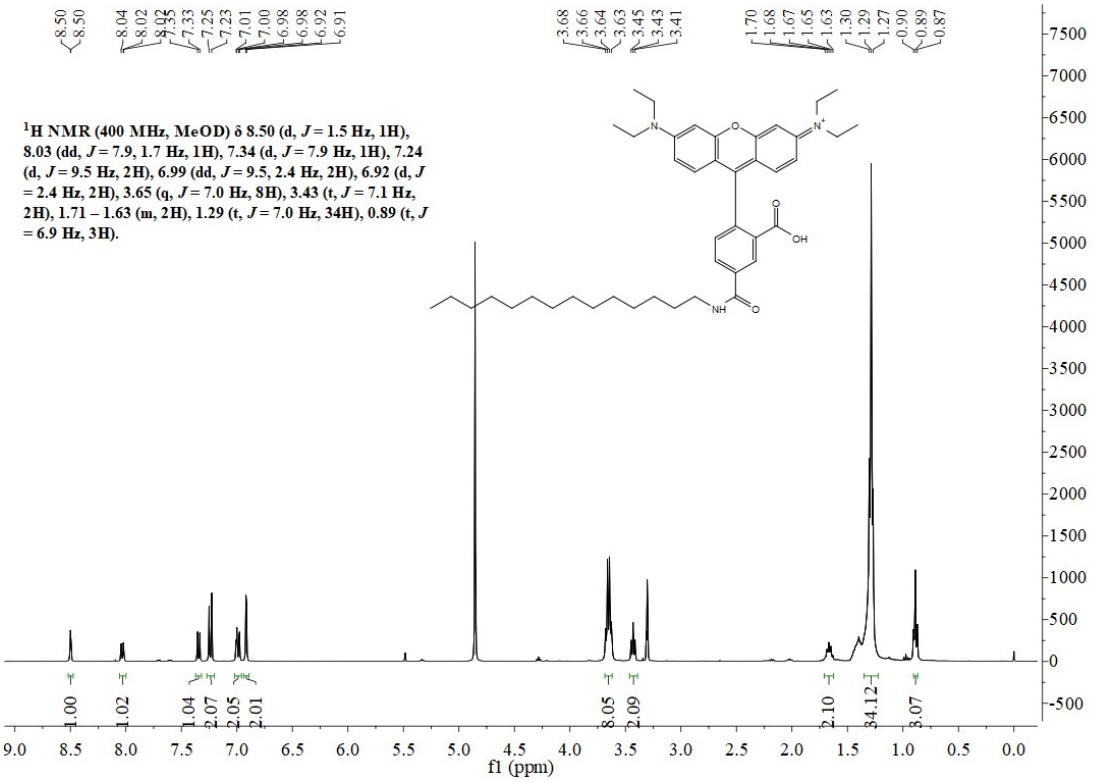


Fig.S11 [1]H NMR spectrum of **RDP** in MeOD.

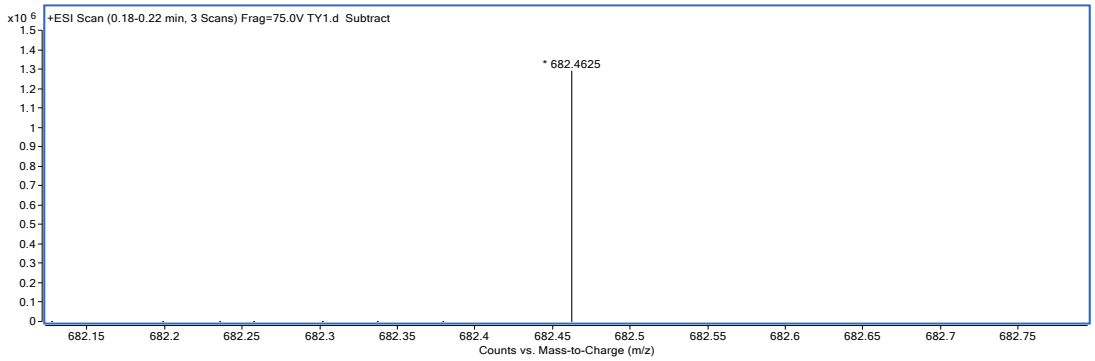


Fig. S12 HRMS of **RDP**.

6 References

1 W. Zhou, X. Fang, Q. Qiao, W. Jiang, Y. Zhang and Z. Xu, *Chinese Chemical Letters*, 2021, **32**, 943-946.

2 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 1959, **37**, 911-917.